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***Short Communication***

**SAL1-PAP retrograde signalling extends circadian period by reproducing the loss of exoribonuclease (XRN) activity**

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## Abstract

Plants have developed an internal timing mechanism, the circadian system, that serves to synchronise physiological and metabolic functions with daily cues such as dawn and dusk, and provides plants with an advantage in adapting to changing and challenging conditions. We have recently shown that the SAL1-PAP-XRN retrograde signalling pathway, which is proposed to regulate plant responses under stress conditions, also acts within the circadian system. Here we provide further evidence of circadian regulation by SAL1-PAP-XRN signalling, thereby affirming a link between molecular timekeeping and abiotic stress response mechanisms.

## TEXT

### *SAL1-PAP retrograde signalling contributes to circadian timing*

Plants are exposed to various environmental changes throughout their lifecycle, and utilise complex integrated mechanisms to sense and adapt to these conditions.<sup>1, 2</sup> Like most organisms, plants have developed an endogenous timing mechanism that serves both to coordinate the various complex biological components of the cell, and to synchronise responses to predictable environmental rhythms generated by the Earth's rotation and orbit <sup>1</sup>.<sup>3</sup> In addition, this circadian clock can allow plants to adapt and predict when abiotic stressors such as drought, high light or frost are likely to occur.<sup>3-5</sup> The oxidative damage caused by environmental stresses are mostly sensed in chloroplasts and mitochondria, and adequate adaptive responses require interorganellar signalling mechanisms.<sup>2</sup> One such mechanism involves the ROS-sensitive enzyme SAL1. *sall* alleles have been identified in multiple mutant screens and have consequently been ascribed several different gene symbols including *ALX8*, *FIERY1*, and *FOU8*.<sup>6-8</sup> SAL1 is localised in chloroplasts and mitochondria and exhibits phosphatase activity towards polyphosphoinositols such as inositol 1,4,5-

triphosphate (IP<sub>3</sub>), and 3'(2')5'-biphosphate nucleotides such as 3'-phosphoadenosine 5'-phosphate (PAP).<sup>7, 9, 10</sup>

Under stress conditions, changes in redox poise in plastids leads to inhibition of SAL1 catalytic activity and subsequent accumulation of PAP in chloroplasts.<sup>10-12</sup> The accumulation of PAP acts as a retrograde signal, affecting expression patterns of plastid redox associated nuclear genes (PRANGs) through inhibition of 5'→3'exoribonuclease (XRN) activity. We have recently shown that the accumulation of PAP observed in response to whole-plant osmotic stress coincides with lengthening of circadian period.<sup>13</sup> Furthermore, loss-of-function *sal1* mutants exhibit elevated PAP levels and long circadian free-running period (FRP) – two mutant phenotypes which are rescued upon overexpression of the PAP-specific SAL1 homologue AHL in *sal1*<sup>13</sup>. SAL1-mediated regulation of both PAP levels and circadian period is further confirmed upon expression of SAL1-GFP under control of its native promoter, with *SAL1::SAL1-GFP (alx8-1)* seedlings exhibiting restored circadian rhythms<sup>13</sup> and PAP levels comparative to wild-type (Fig. 1). Here we further investigate the mechanisms through which SAL1-PAP signalling affects circadian rhythms.

### ***SAL1-PAP-mediated circadian regulation does not occur through secondary sulphur metabolism pathways***

Among the consequences of the *sal1* mutation are low levels of internal sulfate and total elemental sulfur, as well as perturbed secondary sulfate metabolism.<sup>14, 15</sup> While sulfate assimilation is vital for plant metabolism and photosynthesis,<sup>16</sup> we have shown that gross sulfate deprivation does not lengthen circadian period.<sup>13</sup> Similarly, disruption of secondary sulfate metabolism (through mutation of *ARABIDOPSIS 5-PHOSPHOSULFATE KINASE1* [*APK1*] and *APK2*) is insufficient to perturb circadian rhythms in the chloroplast.<sup>13</sup> As

disruption of secondary sulfate metabolism in *apk1 apk2* plants prevents the accumulation of the PAP precursor phosphoadenosine 5'-phosphosulfate we examined the circadian phenotype of *sal1* mutants in an *apk1 apk2* mutant background using chlorophyll fluorescence imaging (Fig. 2<sup>14, 17, 18</sup>). It has previously been shown that PAP accumulation induced by the *fou8* allele of *SAL1* is reduced in *apk1 apk2 fou8* triple mutants<sup>14</sup>. Intriguingly, we observed that this reduction in PAP levels was correlated with a shortening of circadian period (Fig. 2,  $\tau = 23.46 \pm 0.14$  hrs in *apk1 apk2 fou8* compared to  $24.60 \pm 0.13$  hrs in *fou8* seedlings). Such data supports our hypothesis that PAP accumulation underlies the delayed circadian phenotype of *sal1* plants.

### ***SAL1-PAP-XRN signalling regulates circadian rhythms in transcript accumulation***

Despite the highly pleiotropic nature of the *sal1* mutation, the various phenotypes have been attributed mainly to the inhibition of XRN activity by PAP accumulation.<sup>11, 12, 19, 20</sup> In support of this hypothesis we have demonstrated that loss of XRN activity replicates the circadian phenotype of *sal1* mutants.<sup>13</sup> The *xrn2 xrn3 xrn4 (xrn234)* triple mutant exhibits long-period rhythms in  $F_q'/F_m'$ , as well as a delayed phase in transcript accumulation for the morning-phased clock component *CCA1* under constant blue light.<sup>13</sup> A similar phase delay is observed in accumulation for the early-phased clock transcript *LHY* (Fig. 3A) under constant blue light. We also confirm this phase delay in the accumulation of transcripts for evening-phased clock components *PSEUDORESPONSE REGULATOR5 (PRR5)* and *GIGANTEA* under constant blue light (Fig. 3B-C). Interestingly, in both *sal1* and *xrn234*, the phase delay in *CCA1* transcript accumulation was more pronounced under constant blue light than under constant white light.<sup>13</sup> This light-specific phenotype is also observed in transcript accumulation rhythms of *LHY*, *PRR5*, and *GIGANTEA* (Fig. 4 A-C), further supporting the hypothesis that PAP accumulation delays the circadian system through inhibition of XRN activity. Further

investigation of this signalling pathway will require the analysis of higher-order *sall xrn234* plants to better understand the relationship between SAL1 signalling and these downstream exoribonucleases.

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## Figure Legends

### Figure 1

#### **Accumulation of PAP in *sal1* plants.**

PAP levels were determined in Columbia (Col-0), *alx8-1*, and *alx8-1* seedlings transformed with a *SAL1::SAL1:GFP* construct.<sup>13</sup> Plants were grown for 16 days under 12:12 light:dark cycles before being harvested at dawn. Data are the mean of three biological replicates and are representative of two independent experiments.

### Figure 2

#### **Disruption of sulfur catabolism is epistatic to the *sal1* circadian phenotype.**

(A) Outline of sulfate metabolism. Sulfate is incorporated into adenosine phosphosulfate (APS) before APS is used to synthesise phosphoadenosine 5'-phosphosulfate (PAPS). Loss of ARABIDOPSIS PHOSPHOSULFATE KINASE1 (APK1) and APK2 activity is sufficient to limit PAPS and PAP accumulation<sup>14</sup>. (B) Rhythms of PSII operating efficiency ( $F_q'/F_m'$ ) measured over circadian time in *apk1 apk2*, *fou8*, and *apk1 apk2 fou8* seedlings. Plants were grown for 12 d under 12/12-h L/D cycles before being transferred to constant blue light (20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Data represent mean values of multiple seedlings (n = 8) and are representative of three independent experiments. SE is presented every 5 h for clarity. (C) Circadian period estimates of data presented in (B). SE is shown, n = 8.

### Figure 3

#### Circadian phenotypes of *xrn234* plants under constant blue light.

Assessment of *LHY* (A), *PRR5* (B), and *GIGANTEA* (C) circadian transcript accumulation under constant blue light in *Columbia* (Col-0), *alx8-1*, *fry1-6* and *xrn234* seedlings using qRT-PCR. Plants were entrained in 12:12 L/D cycles before being moved to constant conditions with 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$  blue light. Data for each gene were normalized with an internal control (*PP2a*) and are the mean of three biological replicates.

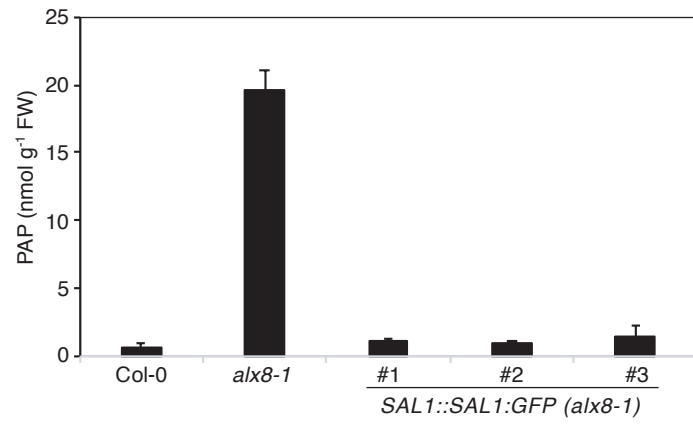
### Figure 4

#### Circadian phenotypes of *xrn234* plants under constant white light.

Assessment of *LHY* (A), *PRR5* (B), and *GIGANTEA* (C) circadian transcript accumulation under constant white light in *Columbia* (Col-0), *alx8-1*, *fry1-6* and *xrn234* seedlings using qRT-PCR. Plants were entrained in 12:12 L/D cycles before being moved to constant conditions with 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white light. Data for each gene were normalized with an internal control (*PP2a*) and are the mean of three biological replicates.



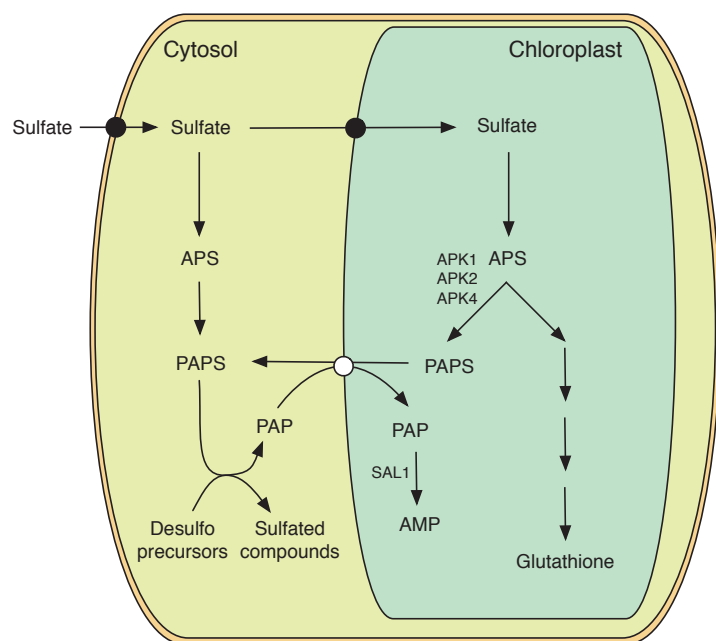
Figure 1



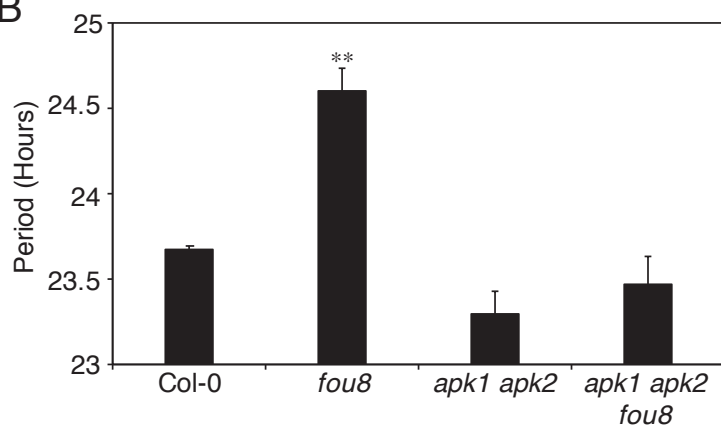
**Figure 1. Accumulation of PAP in *sal1* plants.** PAP levels were determined in Columbia (Col-0), *alx8-1*, and *alx8-1* seedlings transformed with a *SAL1::SAL1:GFP* construct<sup>13</sup>. Plants were grown for 16 days under 12:12 light:dark cycles before being harvested at dawn. Data are the mean of three biological replicates and are representative of two independent experiments. UD, PAP levels were below detection threshold.

Figure 2

A



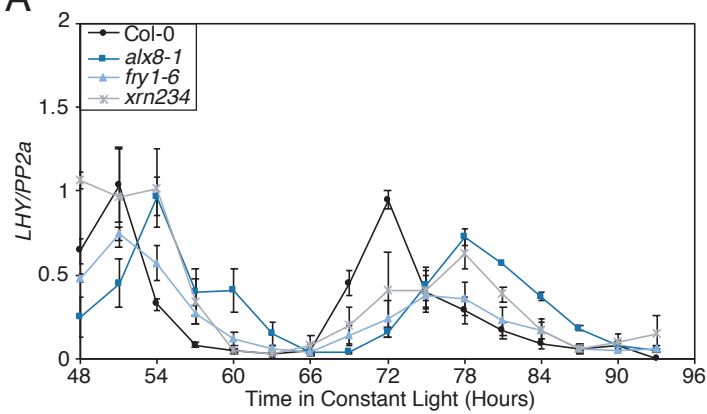
B



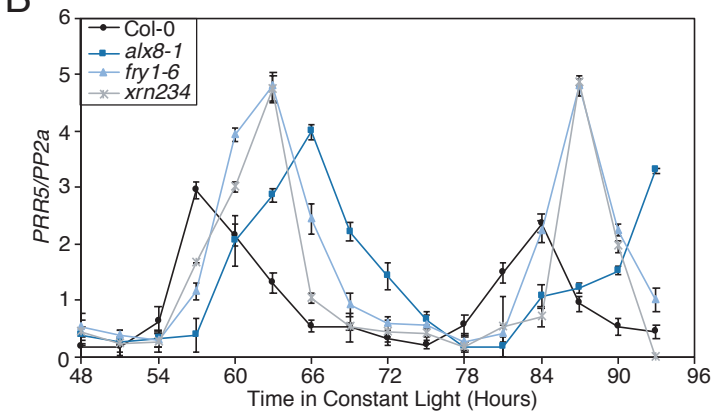
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Figure 3

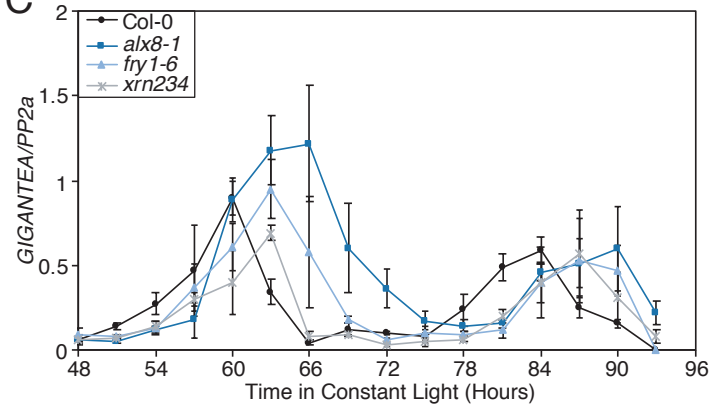
A



B

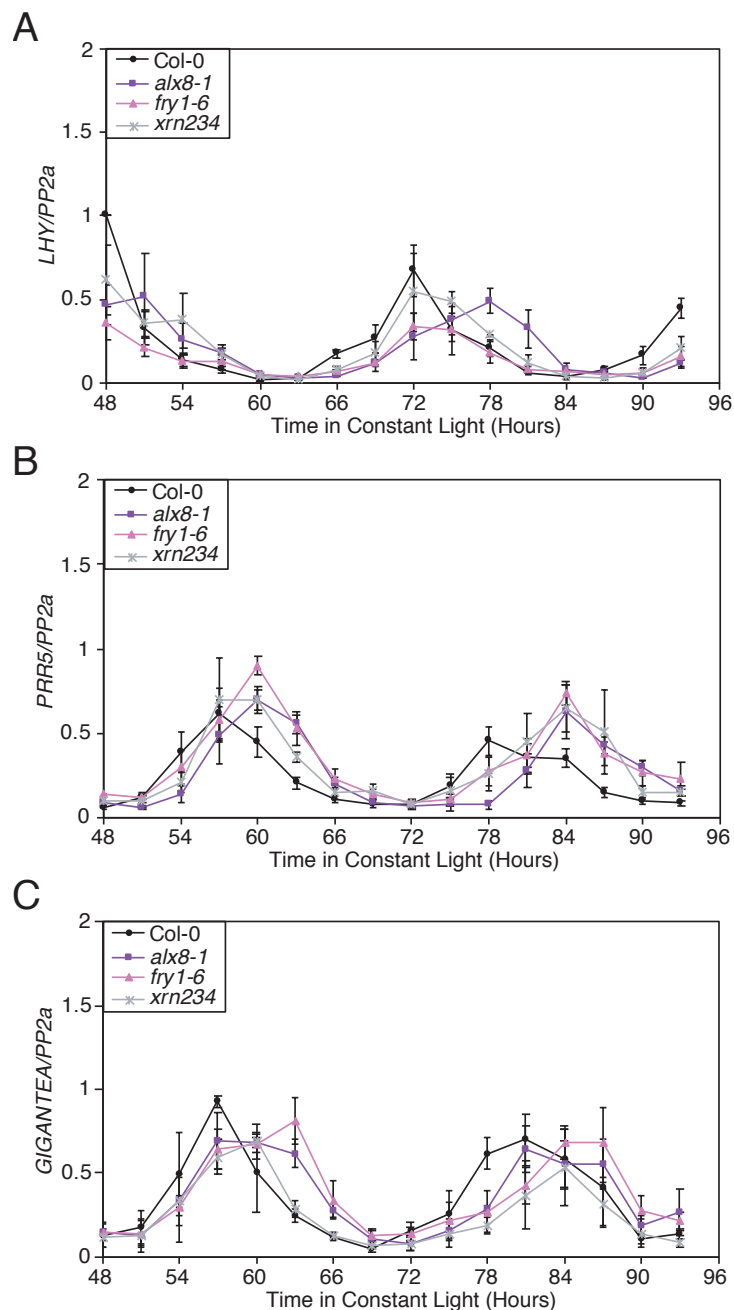


C



**Figure 3. Circadian phenotypes of *xrn234* plants under constant blue light.** Assessment of *LHY* (A), *PRR5* (B), and *GIGANTEA* (C) circadian transcript accumulation under constant blue light in Columbia (Col-0), *alx8-1*, *fry1-6*, and *xrn234* seedlings using qRT-PCR. Plants were entrained in 12:12 L/D cycles before being moved to constant conditions with 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$  blue light. Data for each gene were normalized with an internal control (*PP2a*) and are the mean of three biological replicates.

**Figure 4**



**Figure 4. Circadian phenotypes of *xrn234* plants under constant white light.** Assessment of *LHY* (A), *PRR5* (B), and *GIGANTEA* (C) circadian transcript accumulation under constant blue light in Columbia (Col-0), *alx8-1*, *fry1-6*, and *xrn234* seedlings using qRT-PCR. Plants were entrained in 12:12 L/D cycles before being moved to constant conditions with  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light. Data for each gene were normalized with an internal control (*PP2a*) and are the mean of three biological replicates.